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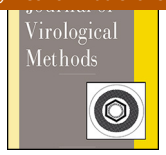

HPV Direct Flow CHIP: A new human papillomavirus genotyping method based on direct PCR from crude-cell extracts☆

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A B S T R A C T

HPV Direct Flow CHIP is a newly developed test for identifying 18 high-risk and 18 low-risk human papillomavirus (HPV) genotypes. It is based on direct PCR from crude-cell extracts, automatic flow-through hybridization, and colorimetric detection. The aim of this study was to evaluate the performance of HPV Direct Flow CHIP in the analysis of 947 samples from routine cervical screening or the follow-up of abnormal Pap smears. The specimens were dry swab samples, liquid-based cytology samples, or formalin-fixed paraffin-embedded tissues. The genotype distribution was in agreement with known epidemiological data for the Spanish population. Three different subgroups of the samples were also tested by Linear Array (LA) HPV Genotyping Test ($n = 108$), CLART HPV2 ($n = 82$), or Digene Hybrid Capture 2 (HC2) HPV DNA Test ($n = 101$). HPV positivity was 73.6% by HPV Direct Flow CHIP *versus* 67% by LA, 65.9% by HPV Direct Flow CHIP *versus* 59.8% by CLART, and 62.4% by HPV Direct Flow CHIP *versus* 42.6% by HC2. HPV Direct Flow CHIP showed a positive agreement of 88.6% with LA ($k = 0.798$), 87.3% with CLART ($k = 0.818$), and 68.2% with HC2 ($k = 0.618$). In conclusion, HPV Direct Flow CHIP results were comparable with those of the other methods tested. Although further investigation is needed to compare the performance of this new test with a gold-standard reference method, these preliminary findings evidence the potential value of HPV Direct Flow CHIP in HPV vaccinology and epidemiology studies.

1. Introduction

Cervical cancer is the third most common cancer among women and the second female cancer-related cause of death worldwide (Jemal et al., 2011). It has been extensively proven that persistent human papillomavirus (HPV) infection is necessary for the development of cervical intraepithelial lesions and invasive carcinoma (Bosch et al., 2002; Walboomers et al., 1999). Although most HPV infections resolve spontaneously, persistence of the so-called high-risk genotypes (Munoz et al., 2003) is directly linked to the malignant progression of the lesions (Kjaer et al., 2002; Remmink et al., 1995; Wallin et al., 1999), with HPV 16 and 18 accounting for approximately 70% of all cervical cancers (IARC, 2005; Munoz et al., 2006).

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HPV testing offers greater sensitivity for the early detection of pre-cancerous cervical lesions in comparison to Pap smears and has been recommended as a triage tool for efficient patient management (Almonte et al., 2011; Saslow et al., 2012; Smith et al., 2006). It has been approved by several countries as the first-line system for cervical cancer screening (Almonte et al., 2011; Anttila et al., 2009; Gustafsson et al., 1997; Peto et al., 2004).

Two different HPV vaccines are currently on the market and are being introduced in several countries as part of their vaccination programs: Cervarix® (GlaxoSmithKline, Rixensart, Belgium), which protects against HPV 16 and 18 (Harper et al., 2006), and Gardasil® (Sanofi Pasteur MSD, Lyon, France), which targets HPV 6, 11, 16, and 18 (Garland et al., 2007). Cross-protection has been observed for other HPV genotypes not included in these vaccines (Ochi et al., 2008; Paavonen et al., 2009; Wheeler et al., 2009). Sensitive detection of the different genotypes is therefore important for evaluating the effectiveness of vaccines and the overall protection provided against HPV. Viral genotyping is also an important tool in studies of the transmission, epidemiology, and natural history of HPV.

Routine HPV detection programs mainly use commercially available methods consisting of either viral DNA capture followed by signal amplification or PCR reactions that specifically target L1-gene viral sequences. The Digene Hybrid Capture 2 (HC2) HPV DNA Test (Qiagen, Gaithersburg, MD) detects 13 high-risk and 5 low-risk genotypes and is based on the binding of cocktail RNA probes to HPV DNA sequences, capturing the RNA-DNA dimmers and amplifying the signal. Other systems are based on L1 amplification followed by reverse dot/line blot hybridization to type-specific probes, such as INNO-LiPA (Innogenetics NV, Gent, Belgium), which uses SPF10 primers and identifies 28 genotypes (Kleter et al., 1999). PGMY primers are also included in the L1 region and different assays based on these are available, including CLART HPV2 (Genomica, Madrid, Spain), which identifies 35 genotypes (Pista et al., 2011), and the Linear Array (LA) HPV Genotyping Test (Roche Molecular Systems, Alameda, CA) which detects 37 genotypes (Castle et al., 2008).

Various HPV detection systems, including HC2, do not identify the specific genotype(s) present in the sample, and their results have a limited usefulness as they can only be reported as high-risk positive, low-risk positive, or high-risk + low-risk positive. On the other hand, sensitive genotype-specific detection systems allow the definition of epidemiological patterns, prognostic predictions based on HPV type-specific infection, the monitoring of viral persistence, and the evaluation of vaccine efficacy and cross-protection (Kjaer et al., 2010; Meijer et al., 2009). These advantages are important for compliance with the new ASCCP guidelines (Saslow et al., 2012), which establish distinct management strategies as a function of genotype, *e.g.*, colposcopy in patients negative for intraepithelial lesion but positive for genotype 16 or 18.

HPV Direct Flow CHIP (Master Diagnóstica, Granada, Spain) is a new commercially available product for sensitive HPV detection and genotyping and is CE-IVD marked in compliance with European Union diagnostic medical device manufacturing standards. The direct PCR system allows [GP5+/GP6+]-based multiplex amplification from crude-cell extracts and does not require previous DNA extraction. The assay detects 18 high-risk or putative high-risk genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82MM4) and 18 low-risk genotypes (6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 67, 69, 70, 71, 72, 81, 84, and 89). The analytical sensitivity of the test was validated after successful participation in the 2011 WHO HPV LabNet Proficiency Study, which demonstrated 100% agreement with reference values in 43 samples evaluated at different concentrations (from 5 to 500 GE) in the setting of single and multiple infections. Furthermore, no cross-reactivity among genotypes was observed; therefore, the analytical specificity for these samples was 100% (manuscript in preparation).

The purpose of the present study was to determine the accuracy of the new test by analysis of 947 cervical samples and comparison with the widely used HC2 system and similar PCR L1-based commercial assays, LA and CLART, investigating the genotype prevalence, infection rates, distribution by diagnostic group, and agreement among methods.

2. Materials and methods

2.1. Sample collection and experimental design

Cervical specimens ($n = 947$) were collected at random during routine gynecological screening or the follow-up of abnormal Pap smears at nine gynecological centers in southern and central Spain from 2010 through 2012. Samples were only eligible for inclusion in the study when: (i) informed consent had been signed, (ii) age and gynecological record were known, (iii) cytopathological and/or histopathological analyses were performed, and (iv) a positive signal for the internal amplification control (human beta-globin) was obtained when tested by HPV Direct Flow CHIP. Pathology reports were compiled and classified according to the Bethesda System for reporting cervical cytology and histology (Solomon et al., 2002) as: negative for intraepithelial lesion (NILM), atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), or cervical carcinoma. All 947 samples were analyzed by HPV Direct Flow CHIP in the laboratories of Master Diagnóstica. The distribution of the different specimens was: 546 dry swabs, 215 liquid-based cytology specimens in PreservCyt® solution (Cytyc, Marlborough, MA, USA); 101 liquid-based cytology specimens in STM transport media, using the Qiagen collection device (Qiagen, Hilden, Germany); and 85 formalin-fixed paraffin-embedded tissue sections.

For comparative analysis with other commercial systems, the following three centers also applied their habitual HPV detection method to their selected samples: (i) Ramón y Cajal Hospital (Madrid, Spain), which analyzed their 108 liquid-based cytology samples in PreservCyt® solution by LA, (ii) 12 de Octubre University Hospital (Madrid, Spain), which analyzed their 82 liquid-based cytology samples in PreservCyt® solution by CLART, and (iii) Virgen del Rocío University Hospital (Sevilla, Spain), which analyzed their 101 liquid-based cytology samples in STM transport media by HC2. In these centers, each sample was divided into two aliquots for subsequent blinded testing: the first aliquot was tested with the corresponding system at the center (for details see Sections 2.2–2.4), and the second aliquot, with unlabelled cell-extracts, was tested by HPV Direct Flow CHIP at the laboratory of Master Diagnóstica, followed by a comparison of the results.

2.2. HPV detection and genotyping by HPV Direct Flow CHIP

For the initial HPV testing, all 947 specimens in the study were analyzed by the HPV Direct Flow CHIP system, which includes a [GP5+/GP6+]-based PCR, automatic reverse dot blot hybridization, and read-out. An additional fragment (268 bp) of the beta-globin gene is co-amplified during the multiplex PCR to assure the quality of the input starting material. The HPV amplification was carried out by direct PCR from crude-cell extracts following the manufacturer's instructions (Master Diagnóstica, Granada, Spain). Briefly, dry swab samples were rinsed in 400 μL sterile phosphate-buffered saline (PBS), the cell suspensions were centrifuged at 110 × g (Heraeus Biofuge Pico- Kendro Laboratory Products, Sollentum, Germany) for 1 min, and the pellets were dissolved in 30–50 μL PBS buffer. For liquid-based cytology samples, the cells were allowed to settle, 200 μL of the suspension were centrifuged and, after rinsing

the pellet in 400 μL PBS, the cells were dissolved in 30–50 μL PBS buffer. Formalin-fixed paraffin-embedded specimens (1–3 sections, 5 μm thickness) were placed in a solution containing 60 μL of Lysis Buffer (Master Diagnóstica, Granada, Spain) with 1.5 μL of DNA Release (Master Diagnóstica, Granada, Spain) and digested in the MJ Mini™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) for 30 min at 60 °C, followed by inactivation at 98 °C for 10 min. For all three types of specimen, the final amplification reaction included 53 μL of the master mix supplied, 1 μL of Phire® Hot Start II DNA polymerase (Thermo Fisher Scientific, Vantaa, Finland), and 6 μL of either crude-cell extract or lysated tissue as DNA template. Amplification cycling conditions in the MJ Mini™ Thermal Cycler were: 5 min of denaturation at 98 °C; 5 cycles of denaturation at 98 °C for 5 s, annealing at 42 °C for 5 s, and elongation at 72 °C for 10 s; 45 cycles of denaturation at 98 °C for 5 s, annealing at 60 °C for 5 s and elongation at 72 °C for 10 s; and final elongation at 72 °C for 1 min. Biotinylated amplicons were denatured for 5 min at 95 °C, cooled in an ice bath for 2 min, and hybridized to HPV CHIP membranes containing immobilized probes for hybridization control, beta-globin gene, HPV-consensus sequences, and genotype-specific HPV detection. The hybridization was performed automatically in sets of 15 samples using the e-BRID System™ (Master Diagnóstica, Granada, Spain), which allows the DNA target molecules to cross the membrane and bind to the complementary probes. Colorimetric detection was carried out by adding NBT-BCIP substrates that detect alkaline phosphatase activity, creating insoluble purple precipitates. The entire process, from the start to the final read-out, was completed in less than three hours.

2.3. HPV detection and genotyping by Linear Array HPV Genotyping Test

The LA HPV Genotyping Test was applied at the Ramón y Cajal Hospital (Madrid, Spain) in 108 liquid-based cytology samples in PreservCyt® solution. The assay qualitatively identifies 37 HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82MM4, 82IS39, 83, 84, and 89). An additional primer pair targeting beta-globin is also included in the LA system as internal amplification control. Tests were performed following the manufacturer's instructions. Briefly, DNA was purified automatically in the COBAS® AmpliPrep device (Roche Diagnostics, Mannheim, Germany), starting from 800 μL of cervical sample. Purified DNA was used as the template to amplify a 450 bp fragment from the L1 viral region in a conventional thermocycler (GeneAmp® PCR System 9700, Applied Biosystems, Foster, CA, USA). Denatured biotinylated amplicons were hybridized to genotype-specific probes in strip membranes using the Profiblot T48 automatic system (Tecan Trading, Männedorf, Switzerland) and Linear Array Detection Kit (Roche Molecular Systems, Alameda, CA, USA). The results were obtained after colorimetric detection and visual analysis of the strips, consulting the reference guide provided.

2.4. HPV detection and genotyping by CLART HPV2

The CLART HPV2 test was applied at the 12 de Octubre University Hospital (Madrid, Spain) to 82 liquid-based cytology samples in PreservCyt® solution (Cytyc, Marlborough, MA, USA). This assay identifies 35 HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68, 70, 71, 72, 73, 81, 82MM4, 83, 84, 85, and 89). The system includes a control for the presence of genomic DNA as well as genotype-specific probes. Samples were processed in accordance with the manufacturer's protocol, and the DNA was purified automatically by using the QIAamp DNA Mini QIAcube kit (Qiagen, Hilden, Germany). In brief, purified DNA was used to perform PGMY-based amplification in a GeneAmp® PCR System 9700 thermocycler, the biotinylated PCR products were hybridized to low-density microarrays, and insoluble precipitates were automatically detected and analyzed by the SAICLART® processing software (Genomica, Madrid, Spain).

2.5. HPV detection by Hybrid Capture 2 HPV DNA Test

The Hybrid Capture 2 HPV DNA Test was applied at the Virgen del Rocío University Hospital (Sevilla, Spain) in 101 samples preserved in STM transport media (Qiagen, Hilden, Germany), following the manufacturer's instructions. This assay detects 13 high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) and 5 low-risk (6, 11, 42, 43, 44) HPV genotypes. Briefly, viral DNA was released by cell lysis, followed by incubation with a cocktail of HPV RNA probes, antibody-mediated dimmer capture, and AP chemiluminescent detection. Specimens with an RLU/CO ratio $\geq$1 were considered positive.

2.6. Statistical analysis

The structure of the data was studied by descriptive analysis. Sample sizes, sample type, HPV positivity, and multiple infection profiles were described. Chi-square tests were used to analyze the relationship of these with age and diagnostic groups. Statistically significant differences, overall agreement, and positive agreement were determined between the results of HPV Direct Flow CHIP and those of LA, CLART, and HC2. Cohen's Kappa coefficient (Landis and Koch, 1977) was also calculated as a measure of the agreement between two methods and as an indication of the accuracy of the classification output after accounting for the random portion. The McNemar chi-square test (Altman, 1999), which analyzes significant differences between assays in matched-pair data, was used to study statistically significant differences between assays in the detection of HPV infections. For the pairwise comparisons, only genotypes identified by both methods were considered for the calculation of agreement. SPSS version 19 (IBM, Chicago, IL, USA) was used for the statistical analyses, which were performed at a 5% significance level.

3. Results

3.1. Study patients and HPV positivity among the 947 samples tested by HPV Direct Flow CHIP

The mean age of all patients studied was 35.5 years (standard deviation [s.d.] of 10.84 years, range 15–90 years). The mean age was 37.3 years (s.d. 10.94) for the HPV-negative patients and 34.4 years (s.d. 10.63) for the HPV-positive patients. The distribution of diagnostic groups was: 45.4% negative for intraepithelial lesions, 21.0% atypical squamous cells of undetermined significance, 24.9% low-grade squamous intraepithelial lesions, 7.2% high-grade squamous intraepithelial lesions, and 1.5% cervical carcinomas.

HPV positivity was detected in 61.5% (582/947) of the samples studied by HPV Direct Flow CHIP. Out of these 582 positive samples, 572 were correctly genotyped, whereas 10 were positive for the HPV-consensus probe but did not hybridize to any genotype-specific probe. HPV infection was significantly age-related, observing the highest HPV infection rate (71.4%) in the youngest age group (<30 years) (chi-square = 20.178, $p < 0.001$). HPV infection rates were significantly higher with greater disease severity, being 37.7% (162/430) in patients negative for intraepithelial lesions, 62.8% (125/199) in patients with atypical squamous cells of undetermined significance, 90.3% (213/236) in those with low-grade squamous intraepithelial lesions, 100% (68/68)

Table 1
Distribution of genotypes identified by HPV Direct Flow CHIP with respect to all HPV positive samples for each diagnosis group. *n*: number of samples detected for the corresponding genotype; %: coefficient of samples positive for the corresponding genotype/all the samples HPV+ included in the group. Cytology category (Bethesda): NILM (negative for intraepithelial lesion or malignancy); ASCUS (atypical squamous cells of undetermined significance); LSIL (low grade squamous intraepithelial lesion); HSIL (high grade squamous intraepithelial lesion); CxCa (cervical carcinoma).

	HPV genotypes																
	6	11	16	18	26	31	33	35	39	40	42	43	45	51	52	53	54
NILM (*n* = 162)																	
N	23	9	36	16	0	10	6	8	4	4	21	8	13	9	15	11	16
%	14.2	5.6	22.2	9.9	0.0	6.2	3.7	4.9	2.5	2.5	13.0	4.9	8.0	5.6	9.3	6.8	9.9
ASCUS (*n* = 125)																	
N	24	6	34	11	2	14	6	8	4	3	5	7	9	5	11	6	6
%	19.2	4.8	27.2	8.8	1.6	11.2	4.8	6.4	3.2	2.4	4.0	5.6	7.2	4.0	8.8	4.8	4.8
LSIL (*n* = 213)																	
N	49	12	94	26	3	27	17	20	12	13	35	18	19	13	30	21	9
%	23.0	5.6	44.1	12.2	1.4	12.7	8.0	9.4	5.6	6.1	16.4	8.5	8.9	6.1	14.1	9.9	4.2
HSIL (*n* = 68)																	
N	13	2	47	4	1	7	2	9	2	2	5	3	4	7	8	1	2
%	19.1	2.9	69.1	5.9	1.5	10.3	2.9	13.2	2.9	2.9	7.4	4.4	5.9	10.3	11.8	1.5	2.9
CxCa (*n* = 14)																	
N	5	2	11	3	0	1	1	1	1	0	4	1	4	0	3	0	0
%	35.7	14.3	78.6	21.4	0.0	7.1	7.1	7.1	7.1	0.0	28.6	7.1	28.6	0.0	21.4	0.0	0.0
Total (*n* = 582)																	
N	114	31	222	60	6	59	32	46	23	22	70	37	49	34	67	39	33
%	19.6	5.3	38.1	10.3	1.0	10.1	5.5	7.9	4.0	3.8	12.0	6.4	8.4	5.8	11.5	6.7	5.7

	HPV genotypes																
	56	58	59	61	66	67	68	69	70	71	72	73	82MM4	84	89	44/55	62/81
NILM (*n* = 162)																	
N	11	6	9	3	7	6	16	1	9	2	0	7	6	3	10	14	16
%	6.8	3.7	5.6	1.9	4.3	3.7	9.9	0.6	5.6	1.2	0.0	4.3	3.7	1.9	6.2	8.6	9.9
ASCUS (*n* = 125)																	
N	5	5	2	1	8	11	9	2	4	2	1	5	4	2	3	26	2
%	4	4	1.6	0.8	6.4	8.8	7.2	1.6	3.2	1.6	0.8	4	3.2	1.6	2.4	20.8	1.6
LSIL (*n* = 213)																	
N	18	12	13	4	32	19	18	5	4	6	3	9	5	8	10	28	11
%	8.5	5.6	6.1	1.9	15.0	8.9	8.5	2.3	1.9	2.8	1.4	4.2	2.3	3.8	4.7	13.1	5.2
HSIL (*n* = 68)																	
N	4	5	2	2	3	3	4	2	1	0	1	1	0	0	3	4	4
%	5.9	7.4	2.9	2.9	4.4	4.4	5.9	2.9	1.5	0.0	1.5	1.5	0.0	0.0	4.4	5.9	5.9
CxCa (*n* = 14)																	
N	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
%	7.1	0.0	0.0	0.0	0.0	7.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.1	0.0
Total (*n* = 582)																	
N	39	28	26	10	50	40	47	10	18	10	5	22	15	13	26	73	33
%	6.7	4.8	4.5	1.7	8.6	6.9	8.1	1.7	3.1	1.7	0.9	3.8	2.6	2.2	4.5	12.5	5.7

in those with high-grade squamous intraepithelial lesions, and 100% (14/14) in cervical carcinoma patients (chi-square = 236.880, $p < 0.001$). There was also a significantly higher presence of high-risk genotypes in HPV-positive samples with greater disease severity (Table 1), ranging from 66.9% in patients negative for intraepithelial lesions, 76.4% in patients with atypical squamous cells of undetermined significance, 84.8% in those with low-grade squamous intraepithelial lesions, 95.6% in those with high-grade squamous intraepithelial lesions to 100% in cervical carcinoma patients (chi-square = 34.294, $p < 0.001$).

The distribution of HPV genotypes is shown in Table 1. Overall, the most prevalent genotypes were HPV 16 (38.1%), HPV 6 (19.6%), HPV 44/55 (12.5%), HPV 42 (12.0%), HPV 52 (11.5%), and HPV 18 (10.3%). The genotype distribution significantly differed among the diagnostic groups (chi-square = 44.215, $p < 0.001$). For example, the presence of HPV 16 and/or HPV 18 rose from 32.1% in patients negative for intraepithelial lesions to 75% in patients with high-grade squamous intraepithelial lesions and 100% in cervical carcinoma patients.

More than one genotype was present in 58.1% of HPV-positive samples. Among these cases, 24.7% harbored 2 genotypes, 11.2% 3 genotypes and 22.2% 4 or more genotypes. The rate of multiple infections also significantly increased with the severity of disease: 51.9% of the patients negative for intraepithelial lesions, 53.6% of the patients with atypical squamous cells of undetermined significance, 66.2% of those with low-grade squamous intraepithelial lesions, 52.9% of those with high-grade squamous intraepithelial lesions, and 71.4% of those with cervical carcinoma (chi-square = 11.137, $p = 0.025$).

3.2. Comparison of HPV detection systems: HPV positivity

HPV positivity rates were significantly higher for HPV Direct Flow CHIP in the paired comparisons: 73.6% HPV Direct Flow CHIP *versus* 67% LA ($p = 0.039$) and 62.4% HPV Direct Flow CHIP *versus* 42.6% HC2 ($p < 0.001$).

In the HPV Direct Flow CHIP *versus* LA set, the results were concordant for 97 samples (70 HPV-positive and 27 HPV-negative by

Table 2
Comparison of HPV positivity for each sub-set. Samples were classified by cytological diagnosis and HPV positivity, percentage of overall and solely positive agreement, Kappa index, and McNemar test. LA, Linear Array HPV Genotyping Test; CLART, GENOMICA CLART HPV2; HC2, Hybrid Capture 2. Cytology category (Bethesda): NILM (negative for intraepithelial lesion or malignancy); ASCUS (atypical squamous cells of undetermined significance); LSIL (low-grade squamous intraepithelial lesion); HSIL (high-grade squamous intraepithelial lesion); CxCa (cervical carcinoma).

	n	HPV Direct Flow CHIP *vs* other systems				Agreement (%)	Positive agreement (%)	Kappa	McNemar*p*-value
		+/+	+/−	−/+	−/−				
HPV Direct Flow CHIP *vs.* LA									
NILM	61	28	7	1	25	86.9	77.8	0.74	0.07
ASCUS	3	1	0	0	2	100	100	1	1
LSIL	30	29	1	0	0	96.7	96.7	–	–
HSIL	12	12	0	0	0	100	100	–	–
CxCa	0	0	0	0	0	–	–	–	–
Total	106	70	8	1	27	91.5	88.6	0.798	0.039
HPV Direct Flow CHIP *vs.* CLART									
NILM	46	21	2	1	22	93.5	87.5	0.870	1
ASCUS	16	8	3	0	5	81.3	72.7	0.625	0.25
LSIL	16	15	1	0	0	93.8	93.8	–	–
HSIL	4	4	0	0	0	100	100	–	–
CxCa	0	0	0	0	0	–	–	–	–
Total	82	48	6	1	27	91.5	87.3	0.818	0.125
HPV Direct Flow CHIP *vs.* HC2									
NILM	73	17	19	0	37	74	47.2	0.48	<0.001
ASCUS	8	6	1	0	1	87.5	85.7	0.6	1
LSIL	14	14	0	0	0	100	100	–	–
HSIL	5	5	0	0	0	100	100	–	–
CxCa	1	1	0	0	0	100	100	–	–
Total	101	43	20	0	38	80.2	68.2	0.618	<0.001

Note: In the "HPV Direct Flow CHIP *vs.* LA" set, 2 samples were excluded from the comparison because the internal amplification control was not positive in the LA test.

both methods) and discrepant for 9 samples; the overall agreement was 91.5%, the concordance for positive samples was 88.6% (Table 2), and the Kappa index showed substantial agreement ($K = 0.798$). Significant differences were found in HPV detection ($p = 0.039$), with 78 positive cases detected by HPV Direct Flow CHIP *versus* 71 by LA. With respect to the diagnostic group, HPV detection was the same by both methods in patients with high-grade squamous intraepithelial lesions, while more HPV-positive cases were detected by HPV Direct Flow CHIP than by LA in patients negative for intraepithelial lesion and in those with atypical squamous cells of undetermined significance. The lowest agreement (<90%) was obtained in patients negative for intraepithelial lesions.

In the HPV Direct Flow CHIP *versus* CLART set, the results were concordant for 75 samples (48 HPV-positive and 27 HPV-negative by both methods) and discrepant for 7 samples (Table 2). Overall agreement was 91.5%, and the Kappa value indicated almost perfect agreement ($K = 0.818$), with no significant difference between HPV Direct Flow CHIP and CLART ($p = 0.125$). The results were highly concordant, especially in the cases with low- and high-grade squamous intraepithelial lesions. More positive samples were detected by HPV Direct Flow CHIP than by CLART in the cases negative for intraepithelial lesions and in the cases with atypical squamous cells of undetermined significance, but this difference was not significant.

The results of the HPV Direct Flow CHIP and HC2 systems were concordant for 43 HPV-positive and 38 HPV-negative samples (Table 2). In the other 20 samples (19 negative for intraepithelial lesions and 1 case of atypical squamous cells of undetermined significance), all were HPV-negative by HC2, whereas 11 were positive for high-risk, 3 positive for high-risk + low-risk, and 6 for low-risk HPV genotypes by HPV Direct Flow CHIP. Out of these 20 discordant cases, 7 were patients with a known history of cervical lesions, HPV persistence, and conization within the previous four years, and these were all high-risk positive by HPV Direct Flow CHIP and HPV-negative by HC2. In addition, 14 cases that were high-risk positive by HC2 were high-risk + low-risk positive by HPV Direct Flow CHIP. Although HC2 detects low-risk HPV 6, 11, 42, 43 and 44 genotypes, one of these genotypes was detected in 7 cases by HPV Direct Flow CHIP but not by HC2.

3.3. Comparison of HPV detection systems: multiple infection rates

Multiple infection rates in positive samples were 61.6% by HPV Direct Flow CHIP *versus* 50.7% by LA and 61.1% by HPV Direct Flow CHIP *versus* 40.8% by CLART (Table 3). HC2 does not distinguish between multiple and single infections and was therefore not included in these comparisons.

Table 3
Comparison of multiple infection rates detected by the three different genotyping methods: DF, HPV Direct Flow CHIP; LA, Linear Array HPV Genotyping Test; and CLART, GENOMICA CLART HPV2.

	n (%), single *vs.* multiple infection detected in each sub-set			
	HPV Direct Flow CHIP *vs.* Linear Array		HPV Direct Flow CHIP *vs.* CLART	
	DF	LA	DF	CLART
Undetermined genotype	1 (1.3%)	0 (0%)	2 (3.7%)	0 (0%)
Single infection	29 (37.2%)	35 (49.3%)	19 (35.2%)	29 (59.2%)
Multiple infection	48 (61.6%)	36 (50.7%)	33 (61.1%)	20 (40.8%)
Two genotypes	21 (26.9%)	15 (21.1%)	16 (29.6%)	11 (22.5%)
Three genotypes	8 (10.3%)	12 (16.9%)	4 (7.4%)	3 (6.1%)
≥Four genotypes	19 (24.4%)	9 (12.7%)	13 (24.1%)	6 (12.2%)

Table 4
Comparison of specific genotype detection, calculating the percentage of samples classified by result, percentages of overall and positive agreement, Kappa index and McNemar test. Italics indicate genotypes that were only detected by one test and therefore not compared. (A) HPV Direct Flow CHIP (DF) *vs.* Linear Array HPV Genotyping Test (LA), calculated for the 69 HPV positive samples (one sample positive for the HPV-consensus probe but negative for all genotypes in the HPV Direct CHIP panel was excluded from this comparison). (B) HPV Direct Flow CHIP (DF) *vs.* GENOMICA CLART HPV2 (CLART), calculated for the 47 HPV positive samples (one sample positive for the HPV-consensus probe but negative for all genotypes in the HPV Direct CHIP panel was excluded from this comparison).

(A)	DF+/LA+		DF+/LA−		DF−/LA+		DF−/LA−		Agreement	Positive agreement	Kappa	Mc Nemar
	n	%	*n*	%	*n*	%	*n*	%	(%)	(%)		*p*-value
6	1	1.45	2	2.9	0	0	66	95.65	97.1	33.33	0.489	0.5
11	1	1.45	1	1.45	0	0	67	97.1	98.55	50	0.66	1
16	18	26.1	3	4.3	0	0	48	69.6	95.65	85.71	0.893	0.25
18	2	2.9	3	4.3	0	0	64	92.8	95.65	40	0.553	0.25
26	0	0	0	0	1	1.45	68	98.55	98.55	–	–	–
31	5	7.25	2	2.9	0	0	62	89.86	97.1	71.43	0.818	0.5
33	3	4.35	1	1.45	3	4.35	62	89.86	94.2	42.86	0.57	0.625
35	2	2.9	5	7.25	0	0	62	89.86	92.75	28.57	0.418	0.063
39	4	5.8	1	1.45	0	0	64	92.75	98.55	80	0.881	1
40	1	1.45	1	1.45	0	0	67	97.1	98.55	50	0.66	1
42	11	15.94	3	4.35	0	0	55	79.71	95.65	78.57	0.854	0.25
43	*0*	*0*	*3*	*4.35*	*0*	*0*	*66*	*95.65*	*95.65*	–	–	–
45	3	4.35	0	0	0	0	66	95.65	100	100	1	1
51	3	4.35	0	0	1	1.45	65	94.2	98.55	75	0.85	1
52	5	7.25	1	1.45	0	0	63	91.3	98.55	83.33	0.901	1
53	8	11.59	2	2.9	2	2.9	57	82.61	94.2	66.67	0.766	1
54	7	10.14	5	7.25	0	0	57	82.61	92.75	58.33	0.698	0.063
56	4	5.8	3	4.35	1	1.45	61	88.41	94.2	50	0.636	0.625
58	4	5.8	0	0	1	1.45	64	92.75	98.55	80	0.881	1
59	2	2.9	2	2.9	2	2.9	63	91.3	94.2	33.33	0.469	1
61	2	2.9	1	1.45	0	0	66	95.65	98.55	66.67	0.793	1
66	5	7.25	2	2.9	2	2.9	60	86.96	94.2	55.56	0.682	1
67	0	0	4	5.8	0	0	65	94.2	94.2	0	–	–
68	2	2.9	4	5.8	1	1.45	62	89.86	92.75	28.57	0.41	0.375
69	0	0	0	0	0	0	69	100	100	–	–	–
70	3	4.35	1	1.45	0	0	65	94.2	98.55	75	0.85	1
71	2	2.9	1	1.45	0	0	66	95.65	98.55	66.67	0.793	1
72	0	0	0	0	0	0	69	100	100	–	–	–
73	1	1.45	0	0	0	0	68	98.55	100	100	1	1
82MM4	1	1.45	1	1.45	0	0	67	97.1	98.55	50	0.66	1
82IS39	*0*	*0*	*0*	*0*	*1*	*1.45*	*68*	*98.55*	*98.55*	–	–	–
83	*0*	*0*	*0*	*0*	*4*	*5.8*	*65*	*94.2*	*94.2*	*0*	–	–
84	3	4.35	0	0	5	7.25	61	88.41	92.75	37.5	0.515	0.063
89	6	8.7	2	2.9	2	2.9	59	85.51	94.2	60	0.717	1
44/55	0	0	3	4.3	0	0	66	95.7	95.65	0	–	–
62/81	9	13.04	5	7.25	1	1.45	54	78.26	91.3	60	0.699	0.219

(B)	DF+/CLART+		DF+/CLART−		DF−/CLART+		DF−/CLART−		Agreement	Positive agreement	Kappa	Mc Nemar
	n	%	*n*	%	*n*	%	*n*	%	(%)	(%)		*p*-value
6	2	4.26	2	4.26	0	0	43	91.49	95.74	50	0.647	0.50
11	0	0	1	2.13	0	0	46	97.87	97.87	0	–	–
16	6	12.77	2	4.26	4	8.51	35	74.47	87.23	50	0.589	0.687
18	2	4.26	1	2.13	0	0	44	93.62	97.87	66.67	0.789	1
26	0	0	0	0	0	0	47	100	100	–	–	–
31	6	12.77	1	2.13	2	4.26	38	80.85	93.62	66.67	0.762	1
33	2	4.26	1	2.13	2	4.26	42	89.36	93.62	40	0.538	1
35	2	4.26	4	8.51	0	0	41	87.23	91.49	33.33	0.466	0.125
39	1	2.13	0	0	0	0	46	97.87	100	100	1	1
40	0	0	0	0	0	0	47	100	100	–	–	–
42	5	10.64	4	8.51	0	0	38	80.85	91.49	55.56	0.669	0.125
43	0	0	2	4.26	0	0	45	95.74	95.74	0	–	–
45	3	6.38	3	6.38	0	0	41	87.23	93.62	50	0.636	0.25
51	3	6.38	1	2.13	0	0	43	91.49	97.87	75	0.846	1
52	5	10.64	0	0	0	0	42	89.36	100	100	1	1
53	5	10.64	1	2.13	0	0	41	87.23	97.87	83.33	0.897	1
54	1	2.13	2	4.26	0	0	44	93.62	95.74	33.33	0.484	0.5
56	2	4.26	0	0	0	0	45	95.74	100	100	1	1
58	3	6.38	1	2.13	2	4.26	41	87.23	93.62	50	0.632	1
59	0	0	0	0	1	2.13	46	97.87	97.87	0	–	–
61	2	4.26	0	0	0	0	45	95.74	100	100	1	1
66	5	10.64	1	2.13	0	0	41	87.23	97.87	83.33	0.897	1
67	*0*	*0*	*5*	*10.64*	*0*	*0*	*42*	*89.36*	*89.36*	–	–	–
68	1	2.13	4	8.51	0	0	42	89.36	91.49	20	0.309	0.125
70	3	6.38	1	2.13	0	0	43	91.49	97.87	75	0.846	1
71	0	0	0	0	1	2.13	46	97.87	97.87	0	–	–
72	1	2.1	1	2.1	0	0	45	95.7	97.87	50	0.657	1
73	1	2.13	1	2.13	0	0	45	95.74	97.87	50	0.657	1
82MM4	0	0	1	2.13	3	6.38	43	91.49	91.49	0	−0.033	0.625

Table 4 (*Continued*)

(B)	DF+/CLART+		DF+/CLART−		DF−/CLART+		DF−/CLART−		Agreement	Positive agreement	Kappa	Mc Nemar
	n	%	*n*	%	*n*	%	*n*	%	(%)	(%)		*p*-value
83	*0*	*0*	*0*	*0*	*2*	*4.26*	*45*	*95.74*	*95.74*	–	–	–
84	1	2.13	0	0	0	0	46	97.87	100	100	1	1
89	0	0	4	8.51	0	0	43	91.49	91.49	0	–	–
44/55	2	4.26	6	12.77	0	0	39	82.98	87.23	25	0.356	0.031
62/81	7	14.89	1	2.13	0	0	39	82.98	97.87	87.50	0.921	1

The McNemar test was used to study the differences between methods in detecting "single infection" or "two or more genotypes". LA and HPV Direct Flow CHIP detected the same genotypes in 19 samples with single infections and in 32 samples with two or more genotypes. However, significantly higher rates of multiple infection were found by HPV Direct Flow CHIP, which detected multiple genotypes in 14 samples identified as single infections by LA ($p = 0.031$). CLART and HPV Direct Flow CHIP detected the same genotypes in 14 samples with single infections and in 18 with two or more genotypes. However, significantly higher rates of multiple infection were detected by HPV Direct Flow CHIP ($p = 0.007$), which evidenced multiple genotypes in 13 samples identified as single infections by CLART.

3.4. Comparison of HPV detection systems: genotype-specific positivity

A good agreement in the genotype distribution was observed between HPV Direct Flow CHIP and the other systems, especially LA (Table 4A and B). However, discrepant results were observed for specific genotypes. Thus, in comparison to LA, HPV Direct Flow CHIP showed a significantly higher detection rate for HPV 18, 35, 44/55, 54, 67, and 68 but a significantly lower detection rate for HPV 33 and 84 genotypes; and, in comparison to CLART, HPV Direct Flow CHIP had a significantly higher detection rate for HPV 6, 35, 42, 43, 44/55, 45, 54, 67, 68, and 89 but a lower detection rate for HPV 16, although statistical significance was not reached.

4. Discussion

This study evaluated the HPV Direct Flow CHIP system, an automatic assay for the sensitive detection and identification of 36 mucosal HPV genotypes. The performance of the new system was studied by analyzing of 947 cervical samples. In addition, three subsets of the studied population were also tested by other commercial methods commonly used in routine HPV detection, and the results were compared.

The most frequent high-risk HPV genotypes detected by HPV Direct Flow CHIP in the 947 cervical samples studied were HPV 16, 52, 18, 31, and 66. The same finding was reported by a recent study that used INNO-LiPA in samples from Spanish women attending cervical cancer screening (Castellsague et al., 2012), despite the difference in HPV testing method and in the amplified L1 region (SPF10 in INNO-LIPA *versus* [GP5+/GP6+]-based in HPV Direct Flow CHIP). The most prevalent high-risk genotypes in the patients negative for intraepithelial lesions were HPV 16, 18, 68, 52, 45, 56, and 31, which is very similar to the distribution described for women with normal cytological findings worldwide (Bruni et al., 2010). Certain genotypes were more frequent in early stages of the disease than in carcinomas and *vice versa*. For instance, HPV 16 was detected in 22.2% (36/162) of HPV-positive patients negative for intraepithelial lesions but in 78.6% (11/14) of HPV-positive cervical carcinoma patients. These data are in agreement with previous reports on the genotype distribution in invasive cervical cancer internationally (Li et al., 2011) and in Spanish populations (Castellsague et al., 2012; Martin et al., 2011).

HPV Direct Flow CHIP yielded significantly higher rates of HPV infection than the other methods used in this study, especially in patients negative for intraepithelial lesions. It should be taken into account that this sub-group included cases with cellular abnormalities other than intraepithelial lesions (*e.g.*, cervicitis, history of abnormal cytology, post-conization patients, *etc.*) and were therefore more prone to harbor viral infections.

HPV Direct Flow CHIP showed a very high concordance with the LA, CLART, and HC2 systems in the pair-matched results (88.6%, 87.3%, and 68.2%; Kappa value 0.798, 0.818, and 0.618, respectively). Despite the high agreement between HPV Direct Flow CHIP and CLART in the 82 liquid-based cytology samples, a pilot comparison in 23 formalin-fixed paraffin-embedded samples demonstrated that HPV Direct Flow CHIP had a much higher sensitivity than CLART in this type of material ($K = 0.233$) (data not shown). Low HPV detection rates in formalin-fixed paraffin-embedded samples have been described for the CLART system (Perez et al., 2012), and they have been attributed to the low quality, cross-linkage, and fragmentation of the input DNA from these materials and to the use of PGMY primers (Biedermann et al., 2004). In the case of the HPV Direct Flow CHIP, the HPV amplicon size (approx. 150 bp) is smaller than in the PGMY-based CLART assay (approx. 450 bp) and the PCR mix is formulated to minimize PCR inhibition by cellular proteases or chemicals in the sample medium or container, even when direct PCR is used with formalin-fixed paraffin-embedded tissue sections, improving the HPV detection rates of HPV Direct Flow CHIP in this type of sample.

Given that the systems mainly differed in the cases negative for intraepithelial lesions and in the cases with atypical squamous cells of undetermined significance and were almost perfect agreement in the cases with high-grade squamous intraepithelial lesions and cervical carcinoma, it can be speculated that HPV Direct Flow CHIP is more effective than the other HPV detection methods in samples with a low viral load. This idea is supported by the excellent analytical performance of the test in the 2011 WHO HPV LabNet HPV DNA proficiency panel. Although the clinical relevance of a low-dose infection has not yet been elucidated, it was an indicator of viral persistence in some of the present patients. Thus, in the sub-set of HPV Direct Flow CHIP *versus* HC2, seven patients with a history of conization and HPV persistence in the previous four years had a normal Pap smear at the time of the study and were HPV-negative by HC2, but they were positive when investigated by HPV Direct Flow CHIP and for the same genotype previously detected in the same patients. This differential outcome of HPV testing may be crucial for the sensitive evaluation of viral persistence/clearance and for the follow-up of patients with a history of abnormal cytology. However, further prospective studies are needed to evaluate the clinical implications of these findings.

HPV Direct Flow CHIP resulted in significantly higher detection rates for 12 genotypes (HPV 18, 35, 44/55, 54, 67, and 68 with respect to LA and HPV 6, 35, 42, 43, 44/55, 45, 54, 67, 68, and 89 with respect to CLART), while LA was more effective in the identification of HPV 33 and 84 and CLART detected two more cases positive for HPV 16. These differences in genotype-specific sensitivity among HPV Direct Flow CHIP, LA, and CLART were also observed in the 2011 WHO HPV LabNet Proficiency Study,

while the four [GP5+/GP6+]-based methods included in the LabNet program (Luminex HPV Genotyping – Multimetrix/Progen; PANArray; Digene HPV genotyping RH Test – Qiagen; and HPV Direct Flow CHIP) showed a very high genotype-specific agreement (manuscript in preparation). Discrepancies in both genotype- and variant-specific detection were expected in the present study, as the tests differ in the viral target sequences for amplification, PCR setup, oligoprobe design, and hybridization conditions. The LA and CLART systems include PGMY-based amplification, while HPV Direct Flow CHIP uses GP5+/GP6+ primers. Thus, the three methods used in the present study are based on broad-spectrum primers instead of genotype-specific amplification, producing competition and the preferential amplification of certain genotypes due to distinct primer-target affinities, especially in low viral load and/or multiple infection scenarios (Gillio-Tos et al., 2006).

In addition, the multiple infection rates were higher in HPV Direct Flow CHIP than in LA or CLART, reflecting its higher analytical sensitivity. This improved detection of multiple genotypes per sample has already been observed for other highly sensitive HPV detection systems (Garcia et al., 2011; Schmitt et al., 2010; Soto-De Leon et al., 2011), and their presence has been proposed as a possible biomarker of cervical dysplasia progression (Li et al., 2011), although further research is required. In the present study, the multiple infection rate was higher with a more severe diagnosis, as documented in a Columbian population screened using a highly sensitive multiplex PCR and Luminex system (Garcia et al., 2011).

Finally, this study demonstrated the good performance of the HPV Direct Flow CHIP system for HPV testing in different types of sample, using crude-cell extracts as the DNA template for amplification. It is important to note that the lack of a reference method for general comparison and the use of three different groups for pairwise analysis limit the conclusions that can be drawn from this study, and the performance of this system has yet to be thoroughly examined. Surveys in larger populations that include intra- and inter-laboratory determinations and comparisons with a gold-standard method are required for the optimal characterization of this test.

In conclusion, comparative results obtained in this pilot study demonstrated that the performance of HPV Direct Flow CHIP is similar to that of LA, CLART, and HC2. Given that it offers direct PCR from clinical specimens without a DNA purification step, this novel test may be a valuable tool for automated, rapid, and sensitive HPV genotyping, especially in large-scale vaccine surveillance and epidemiology studies.

Conflict of interest

The author EHH is an employee of Master Diagnóstica.

Ethical approval

All study procedures complied with national legislation on data protection and were approved by the clinical research ethics committees of the participating hospitals.

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